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CATECHOL OXIDASE OF RED DELICIOUS APPLE PEEL

DAVID A. STELZIG, S. AKHTAR and S. RIBEIRO

Division of Plant Science, West Virginia University, Morgantown, West Virginia, 26506, U.S.A.

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Abstract—Catechol oxidase has been solubilized from the particulate fraction of apple peel using Triton X-100, dialysis and butanol extraction. The partially purified enzyme retained activity during lyophilization in the presence of mercaptobenzothiazole. The substrate specificity and pH optima of the enzyme are reported.

INTRODUCTION

THE ISOLATION of catechol oxidase (*o*-diphenol: O_2 oxidoreductase, 1.10.3.1) is accompanied by difficulties not always associated with the isolation of other plant enzymes. Plant tissues which have a high catechol oxidase activity also usually contain an abundance of phenolic substrates. The enzymatic and non-enzymatic oxidation of these phenols in the presence of molecular oxygen produces quinones, which can rapidly denature many enzymes[1–3] including catechol oxidase.[4,5] In addition, although catechol oxidase is frequently soluble or easily solubilized, many plants contain particulate catechol oxidases which are extremely difficult to solubilize.[6–8]

Various methods of extraction have been described in the literature in at attempt to minimize the denaturation of catechol oxidase resulting from phenol oxidations.[5] Ascorbic acid or some other antioxidant is frequently incorporated into the homogenizing solution.[9] Organic solvent extraction has been utilized as the first step of purification in an attempt to quickly separate the enzyme from the phenols.[10] Catechol oxidase was recently isolated from potato by a method in which the first step was an ammonium sulfate precipitation of the enzyme from a solution containing benzoic acid, a competitive inhibitor of potato catechol oxidase.[11]

Harel *et al.*[7] demonstrated that Grand Alexander apples contain a mitochondrial catechol oxidase which they were unable to solubilize, and at least three chloroplast catechol oxidases which readily solubilized in the presence of Triton X-100, a nonionic detergent. The catechol oxidase of Cox's Orange Pippin apples was easily solubilized with this same detergent,[12]

[1] M. LIEBERMAN and J. B. BIALE, *Plant Physiol.* **31**, 420 (1956).
[2] C. R. SLACK, *Phytochem.* **5**, 397 (1966).
[3] D. M. STOKES, J. W. ANDERSON and K. S. ROWAN, *Phytochem.* **7**, 1509 (1968).
[4] L. L. INGRAHAM, *J. Am. Chem. Soc.* **77**, 2875 (1955).
[5] J. W. ANDERSON, *Phytochem.* **7**, 1973 (1968).
[6] J. R. L. WALKER, *Austral. J. Biol. Sci.* **17**, 360 (1964).
[7] E. HAREL, A. M. MAYER and Y. SHAIN, *Phytochem.* **4**, 783 (1965).
[8] A. M. MAYER, *Phytochem.* **5**, 1297 (1966).
[9] A. M. MAYER, E. HAREL and Y. SHAIN, *Phytochem.* **3**, 447 (1964).
[10] C. T. SHANNON and D. E. PRATT, *J. Food Sci.* **32**, 479 (1967).
[11] K. BALASINGAM and W. FERDINAND, *Biochem. J.* **118**, 15 (1970).
[12] J. R. L. WALKER and A. C. HULME, *Phytochem.* **5**, 259 (1966).

although previous attempts to solubilize this enzyme had failed.[13] Other reports have also indicated difficulties in solubilizing particulate apple catechol oxidase.[6,10,14]

This paper describes a procedure of extracting apple catechol oxidase with little or no phenol oxidation, and a procedure of solubilizing this tightly bound enzyme.

RESULTS AND DISCUSSION

Since catechol oxidase activity is substantially higher in the peel than in the flesh of the apple,[15] Red Delicious apple peel was chosen as the enzyme source. It was felt that flash-frozen, lyophilized and powdered peel was a significantly better source of enzyme than the fresh peel for two reasons; (a) the powder provided a uniform supply of enzyme for all the extractions, and (b) this procedure removed essentially all of the water from the peel by a method which prevented any significant amount of phenol oxidation. The catechol oxidase activity of the powder was tested and shown to be equivalent to the activity in the fresh peel.

To minimize enzyme denaturation by phenol oxidation products, the powder was homogenized in the presence of both mercaptobenzothiazole (MBT) and cysteine. Extraction with either of these chemicals alone result in a lower catechol oxidase activity (Table 1).

TABLE 1. THE RELATIVE QUANTITIES OF CATECHOL OXIDASE EXTRACTED FROM LYOPHILIZED RED DELICIOUS APPLE PEEL BY VARIOUS HOMOGENIZING SOLUTIONS*

Addition to homogenizing solution	Relative catechol oxidase activity (μl O_2/ml/min)	Color of extract
2×10^{-4} M MBT + 0·2% cysteine	100	Light green
2×10^{-4} M MBT	71	Light green
0·2% cysteine	69	Light brown-green
2×10^{-4} M MBT + 0·01 M ascorbic acid	91	Light green
0·01 M ascorbic acid	62	Brown-green
None	31	Dark brown

* The peel was homogenized in 0·1 M phosphate buffer, pH 6·0, containing 0·3 M sucrose and the reagents indicated. After centrifugation at 20,000 *g* for 30 min , the pellet was washed once with phosphate buffer, resuspended in this buffer and assayed.

MBT is a potent noncompetitive inhibitor of catechol oxidase, but probably has little effect on the nonenzymatic oxidation of phenols.[16] Cysteine reacts with quinones, forming products which are unable to inhibit catechol oxidase.[5] It can also be seen from Table 1 that in this system cysteine might have been a more efficient protector of the enzyme than the commonly used ascorbic acid.

Since all of the detectable catechol oxidase was particulate, the tissue homogenate was subjected to a high speed centrifugation and the pellet washed with buffer. The resuspended pellet was treated separately with a large number of detergents in an attempt to solubilize the catechol oxidase. Of these, only Triton X-100 released substantial amounts of the catechol oxidase into a clear, green supernatant.

[13] J. R. L. WALKER and A. C. HULME, *Phytochem.* **4**, 677 (1965).
[14] J. S. CHALLICE and A. H. WILLIAMS, *Phytochem.* **9**, 1261 (1970).
[15] E. HAREL, A. M. MAYER and Y. SHAIN, *Physiol. Plantarum* **17**, 921 (1964).
[16] J. K. PALMER and J. B. ROBERTS, *Science* **157**, 200 (1967).

Originally, attempts to purify the catechol oxidase released by the detergent treatment were unsuccessful. The enzyme was irreversibly salted out with ammonium sulfate. It adsorbed at the top of a DEAE cellulose column and could not be eluted, even with pH 9 buffer saturated with sodium chloride. The enzyme activity was eluted shortly after the solvent front from an agarose gel column in the same tubes as the green color. It had to be assumed that the Triton X-100 did not truly solubilize the catechol oxidase. This conclusion is similar to that reached by Harel *et al.*[7] They discovered that the catechol oxidase released from a mitochondrial preparation of Grand Alexander apples by a digitonin treatment failed to move from the origin in starch or agar gel electrophoresis at various pH values.

TABLE 2. A SUMMARY OF THE PURIFICATION OF CATECHOL OXIDASE FROM RED DELICIOUS APPLE PEEL

Fraction	Volume (ml)	Activity (μl O_2/min/ml)	Total activity (μl O_2/min)	Protein (mg/ml)	Specific activity (μl O_2/min/mg)	Yield (%)
Particulate	100	4·3	430	0·94	4·6	100
Triton X-100 treated (dialyzed)	120	13·6	1632	0·44	31	380
Butanol extracted	63	23·9	1506	0·18	133	350
Butanol extracted (dialyzed)	87	24·4	2123	0·094	258	495
Calcium phosphate gel fractionated (dialyzed)	110	8·4	924	0·001	8400	215
Lyophilized (dialyzed)	127	7·1	902	0·001	7100	210

When the Triton X-100 treated enzyme was dialyzed and then extracted with *n*-butanol, the green color was removed and the resulting catechol oxidase was probably truly solubilized. When dialysis was omitted, all of the catechol oxidase activity was located in a white precipitate which accumulated at the water–butanol interface during centrifugation. Attempts to redissolve this precipitate were unsuccessful. Butanol extraction without detergent treatment did not release the catechol oxidase activity from the high speed pellet.

The aqueous phase after butanol extraction was dialyzed overnight against water. The catechol oxidase was further purified using calcium phosphate gel adsorption. The resulting enzyme preparation was highly purified (Table 2) and an attempt was made to stockpile the enzyme by lyophilization. Attempts to lyophilize the enzyme without prior dialysis against water resulted in nearly complete denaturation. Even after dialysis, the enzyme could only withstand lyophilization if it was stabilized by the addition of MBT. This necessitated another dialysis before the properties of the lyophilized enzyme could be studied. A summary of this purification is given in Table 2. It is obvious from this table that the high specific activity of the purified catechol oxidase is largely due to the increase in the total activity during the purification steps. Perhaps solubilization and removal of phenols is responsible for this increase.[11]

Both the particulate and the highly purified catechol oxidase preparations were assayed over a pH range from 3·5 to 7·5 using 4-methylcatechol as the substrate. This substrate was dissolved in 0·1 M citrate–phosphate buffer between pH 3·5 and 6·0 and in 0·1 M phosphate buffer from pH 6·0–7·5. The results were corrected for the auto-oxidation of the substrate at pH values above 6·0.

The particulate enzyme had one pH optimum at 5·1 and another at 7·0, while the purified enzyme preparation had pH optima at 4·2 and 7·0. The activity at the lower pH optimum was slightly higher than the activity at pH 7·0 with either enzyme preparation.

Harel *et al.*[7] studied the catechol oxidases of Grand Alexander apples. They demonstrated that the enzymes from the chloroplasts had an optimum of pH 5·1 and that the enzymes from the mitochondria had a pH optimum of 7·3. The chloroplast enzyme was readily solubilized with Triton X-100, but the mitochondrial enzyme could not be truly solubilized. The catechol oxidases of Red Delicious apples are similar to those of Grand Alexander apples in that two pH optima are exhibited, but differ in that neither enzyme is solubilized by detergent treatment alone.

Table 3 demonstrates that at either pH 4·2 or 7·0 the most rapidly oxidized substrate

TABLE 3. A SUMMARY OF THE EASE OF OXIDATION OF VARIOUS PHENOLS AT pH 4·2 AND 7·0 IN THE PRESENCE OF CATECHOL OXIDASE PURIFIED FROM RED DELICIOUS APPLE PEEL

Phenol	Enzyme activity (μl O_2/min/ml)	
	pH 4·2	pH 7·0
4-Methylcatechol	9·8	9·5
Chlorogenic acid	8·8	8·5
Catechol	4·2	3·8
(+)-Catechin	3·2	3·0
Quercetin	0·4	0·4
Tyrosine	0·2	0·2
Quinol	0·0	0·0

of those tested is 4-methylcatechol. Chlorogenic acid is nearly as good a substrate and is very likely the major *in vivo* substrate. There is little or no laccase activity in this preparation as evidenced by the inability to catalyze the oxidation of quinol. The almost negligible tyrosine oxidation may be due to a peroxidase contaminant or may represent a true property of the catechol oxidase. With the exception of catechin, it appears that the flavonoid-like compounds are very poor substrates of the enzyme.

Balasingam *et al.*[11] have reported that the catechol oxidase of King Edward potato tubers is a ribonucleoprotein, and that the RNA component is essential for enzyme activity. Purified catechol oxidase from Red Delicious apple peel has an ultraviolet spectrum typical of a nucleoprotein and gives a positive response to the orcinol test. It is not sensitive to bovine pancreatic ribonuclease, nor is it a substrate of this enzyme. If the purified apple enzyme is precipitated with 5% trichloroacetic acid, the supernatant contains a non-dialyzable, orcinol-positive substance, which has an ultra-violet spectrum identical to yeast RNA, and is a substrate of ribonuclease (as indicated by a time-dependent decrease in the 300 nm adsorbance when the two are mixed). Purified Red Delicious catechol oxidase may be a ribonucleoprotein. However, it is equally possible that the purified preparation merely contains a co-precipitate of RNA.

Ingraham[4] has presented evidence that catechol oxidases from a variety of sources are rapidly inactivated in the presence of substrate phenols, even if sufficient ascorbic acid is present to prevent discoloration of the solutions. Apparently the steady state concentration

of quinones, which forms in the presence of this antioxidant, is sufficient to denature catechol oxidase.

The procedure described is significant in that extraction of catechol oxidase from a tissue rich in phenolic compounds is accomplished with a minimum of phenol oxidation. Very likely the use of MBT and cysteine, and frequently an initial lyophilization step, is widely applicable for the extraction of labile chemicals from tissues rich in phenols and catechol oxidase.

The catechol oxidase of Red Delicious apple peel is tightly bound in the particulate fraction, probably to a lipoprotein structure. Evidently the detergent treatment removed a portion of the nonenzymatic material, but true solubilization was only achieved when the detergent treated enzyme was extracted with butanol. Alternately, the detergent may have temporarily solubilized the enzyme, but the lipid and enzyme reaggregated as the detergent was removed during subsequent steps. If so, it was necessary to remove the lipid by an organic solvent extraction to insure that the lipid and enzyme would not reaggregate.

It is possible that other plant enzymes, which have been reported as difficult to solubilize, could be solubilized by the procedure described in this paper.

EXPERIMENTAL

Enzyme source. Red Delicious apples were harvested approximately 1 month prior to maturity and peeled by hand directly into a Dewar flask containing liquid N_2. The frozen peel was lyophilized, ground in a Wiley Mill using a 40 mesh screen and stored at −20°.

Enzyme extraction and purification. The lyophilized powder (2 g) was homogenized at 4° in 100 ml of 0·1 M phosphate buffer, pH 6, containing 0·3 M sucrose, 0·2% cysteine hydrochloride and 1 ml of 2×10^{-2} M MBT in 95% EtOH. The homogenate was centrifuged at 20,000 *g* for 20 min and the supernatant discarded. The pellet was washed with 0·1 M phosphate buffer, pH 6·0, containing 0·2% cysteine HCl and resuspended in this buffer. This suspension was made 2% with respect to Triton X-100, incubated at 25° for 15 min and centrifuged at 40,000 *g* for 30 min. The pellet was discarded and the supernatant dialyzed overnight against H_2O. The dialysate was extracted with −20° *n*-butanol and dialyzed. The enzyme was adsorbed on calcium phosphate gel (Bio-Rad, lot 9230) using 0·34 ml of gel/100 μg protein. The enzyme was eluted from the gel with 0·1 M phosphate buffer, pH 7·6 containing 5% $(NH_4)_2SO_4$, dialyzed overnight against H_2O and lyophilized in the presence of 2×10^{-4} M MBT. The bulk of the MBT was removed from the redissolved enzyme by centrifugation. The remainder was removed by dialysis.

Enzyme assays. Enzyme assays were performed at 30° in a Gilson Oxygraph equipped with a Clark electrode. Enzyme was added to a solution containing 0·25 ml of 0·1 M 4-methylcatechol and sufficient 0·1 M phosphate buffer, pH 6·0, to give a total vol. of 1·25 ml. The initial rate of oxidation was used to calculate the amount of O_2 utilized. An identical assay was utilized in determining the pH optima, except that the buffer was 0·1 M phosphate from pH 3·5 to 6·0 and 0·1 M citrate–phosphate from pH 6·0 to 7·5. The specificity of the enzyme preparations was assayed in a manner exactly analogous to the routine assay except with the sparingly soluble phenols, tyrosine and quercetin. The catechol oxidase activity was measured in saturated solutions of these phenols.

Protein assay. Protein was determined according to the method of Lowry *et al.*[17] using bovine serum albumin as the standard.

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[17] H. O. Lowry, N. J. Rosebrough, A. L. Farr and R. F. Randall, *J. Biol. Chem.* **193**, 265 (1951).

Key Word Index—Pyrus malus; Rosaceae; apple; phenolase; enzyme extraction.